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(54) Title: EXPRESSION OF PROTEINS ON BACTERIAL SURFACE
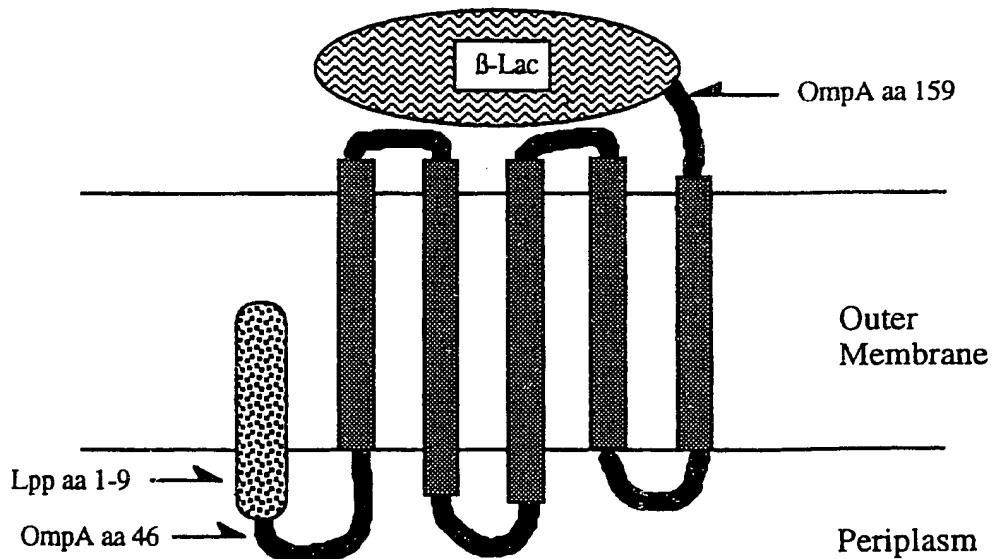
(57) Abstract

The invention relates to a method for producing stable, surface-expressed polypeptides from recombinant gram-negative bacterial cell hosts. A tripartite chimeric gene and its related recombinant vector include separate DNA sequences for directing or targeting and translocating a desired gene product from a cell periplasm to the external cell surface. A wide range of polypeptides may be efficiently surface expressed, including β-lactamase and alkaline phosphatase. Full enzyme activity is maintained and the proteins remain anchored to the bacterial outer membrane surface.

DESCRIPTION

EXPRESSION OF PROTEINS ON BACTERIAL SURFACE

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates generally to the exportation
and localization of polypeptides to the external membrane
surface of a gram-negative cell, to recombinant vectors
useful for the transformation of a host cell and to
chimeric genes that provide outer membrane targeting and
transmembrane sequences. Methods are disclosed providing
for surface expression of proteins, including
antigenically active proteins, specific binding proteins
and enzymatically active species.

Description of Related Art

There is substantial interest in the expression of
selected proteins on the surface of bacteria. Many
potential applications exist, including the production of
genetically engineered whole cell adsorbents,
construction of "peptide libraries" where bacteria carry
different exposed sequences, cell-bound enzymes (another
form of immobilization), and use as live vaccines or
immunogens to generate antibodies.

One approach to obtaining surface expressed foreign
prot ins has been to use a native membran prot in as a
carrier for a foreign protein. LamB, an outer membrane

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protein of *Escherichia coli*, has been fused with peptides
varying in length up to about 60 amino acids with
successful expression of the hybrid protein at a
recombinant host cell surface (Charbit, *et al.*, 1991).

5   Unfortunately, only relatively short polypeptides are
surface-expressed using this method. Outer membrane
proteins have "loop" regions spanning the membrane
surface and while it is possible to substitute foreign
DNA into the gene regions encoding the loop regions,

10 there are only a limited number of insertions possible,
constrained by the size of the loop region and,
apparently, by the requirement to preserve the
penetration and translocating properties of the membrane
protein.

15

In general, attempts to develop methods of anchoring
larger proteins as well as the smaller peptides on a
bacterial cell surface have focused on fusion of the
desired recombinant polypeptide to a native protein that

20 is normally exposed on the cell's exterior in hope that
the resulting hybrid will also be localized on the
surface. The problem with this approach is that fusion
of the foreign protein interferes with localization and,
in many cases, the hybrid molecule is unable to reach the

25 cell surface.

Nevertheless, in one example employing the
Klebsiella enzyme pullulanase, a normally periplasmic
protein, ß-lactamase, was translocated through the *E.*

30 *coli* outer membrane. C-terminal regions of pullulanase
were replaced with DNA segments encoding ß-lactamase or
alkaline phosphatase. Only the hybrid protein with ß-
lactamase was transported to the cell surface (Kornacker
and Pugsl y, 1990). However, the surface-expressed

35 protein was only transiently anchored to the cell
surface, suggesting a severe limitation on the pot ntial
value of any other proteins xpressed by this method as

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surface immunogens, adsorbents, or surface immobilized
species. Furthermore, the assembly of pullulanase
fusions onto the cell surface is a very complicated
process requiring the presence of at least 14 foreign
5   gene products in the host cell. It should be noted that
alkaline phosphatase fused to the same pullulanase
sequence could not be localized on the cell surface
(Kornacker and Pugsley, 1990).

10   The mechanisms of protein insertion within- and
translocation across- the outer membrane of gram-negative
bacteria are not well understood. For some outer
membrane proteins, such as the PhoE porin, the
information necessary for proper localization and
15   assembly is interspersed within the primary sequence
(Bosch et al., 1986; Bosch et al., 1989). Alternatively,
the targeting signal may be contained within a single
short continuous segment. For example, the first nine N-
terminal amino acids of the major E. coli lipoprotein are
20   necessary for proper localization in the outer membrane.
Fusion to this short sequence is sufficient to direct the
normally soluble periplasmic protein ß-lactamase to the
outer membrane (Ghrayeb and Inouye, 1984). Similarly,
extensive studies with OmpA have suggested that the
25   region between residues 154 and 180 is crucial for
localization (Klose et al., 1988a, 1988b). With OmpA,
targeting and outer membrane assembly appear to be
distinct events. Only large fragments containing the
entire membrane spanning sequence of OmpA are able to
30   assemble into a conformation exhibiting native resistance
to proteolytic digestion (Klose et al., 1988a).

In general, amino acid substitutions or insertions
within outer membrane loops exposed on the cell surface
35   are well tolerated and do not interfere with th folding
of the protein in the membrane. Peptides as larg as 60
amino acids have been inserted within external loops of

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various outer membrane proteins and appear to be exposed
n the surface of intact *E. coli* cells as indicated by
immunochemical techniques (Charbit et *al.*, 1991).
However, efforts to direct soluble reporter proteins such
as alkaline phosphatase, to the cell surface using outer
membrane protein fragments have not been successful.
These fusions either end up at incorrect cellular
locations or become anchored in the membrane with the
secreted protein domain facing the periplasm (Murphy *et
al.*, 1990). In gram-negative bacteria the outer membrane
acts as a barrier to restrict the export of proteins from
the cell. Normally only pilins, flagellins, specific
enzymes and a few toxins are completely transported
across the outer membrane (Kornacker and Pugsley, 1990).
Most of these proteins are first secreted into the
periplasmic space via the general secretion pathway and
then cross the outer membrane by a process that involves
the action of several additional gene products (Filloux
et *al.*, 1990).

      Whole cell adsorbents are considered to have
potential value for biotechnology applications for the
purification of various molecules or the selective
removal of hazardous compounds from contaminated waste
waters. However, a major constraint in the development
of whole cell adsorbents is the availability of bacterial
strains with suitable ligands on their surface. Although
functional antibody fragments have been produced in
Escherichia coli (Skerra and Pluckthun 1988, Better et
al. 1988, Orlandi et *al.* 1989, Sastry et *al.* 1989), these
polypeptides have not been expressed on the cell surface.
Indeed, a "library" of recombinant immunoglobulins
containing both heavy and light variable domains (Huse et
al. 1989) has been produced with the proteins having
antigen-binding affinity comparable to th corresponding
natural antibodies. Furthermore, the variety of
recombinant immunoglobulins from bacteria is greater than

the number of antibody molecules that can be generated by
the mammalian cell. In this way it has become possible
to expand the repertoire of antibodies that can be made
by the immune system (Huse et al. 1989). While the
5   availability of such a wide range of immunoglobulins
suggests the potential for creation of E. coli cells
endowed with immunological surface receptors, there has
been little success in producing recombinant proteins on
the surface of bacterial cells, and conspicuous lack of a
10   method to generate recombinant immunoglobulins on
surfaces of gram negative host cells.

     Although the potential repertoire of immunoglobulins
produced in an immunized animal is high ($>10^{10}$), only a
15   small number of monoclonal antibodies can be generated
using hybridomas. This limitation complicates the
isolation of antibodies with specific properties, such as
the ability to act as a catalyst. Combinational antibody
libraries comprising millions of genes of different
20   antibodies have been cloned using phage λ (Huse et al.,
1989). However, screening the library to select the
desired clone can be extremely time consuming and
complicated. One approach to the screening problem has
been an attempt to express antibodies on the surface of
25   filamentous phage. Phage particles displaying high
affinity antibody molecules on their surface can be
enriched by chromatography through a column of
immobilized antigen (Barbas et al., 1991; Clarckson et
al., 1991; Breitling, 1991). Although the feasibility of
30   this technique has been demonstrated, several problems
are apparent, including: (1) fusion to bacteriophage coat
proteins causing interference with antibody folding, (2)
subcloning of large numbers of positive phage particles
in order to produce soluble antibody fragments to carry
35   ut more extensive characterization, and (3) lack of
control of the number of antibody molecules on the phage

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surface, thus affecting binding to the immobilized antigen and complicating the selection procedure.

SUMMARY OF THE INVENTION

5   The present invention addresses one or more of the foregoing problems in providing a versatile recombinant vector that will promote transport of a periplasmic or other protein to the external face of the outer membrane

10   of a gram-negative bacterial cell in the absence of any specific export components. In particular, the vector includes a tripartite chimeric gene having a membrane targeting sequence, a membrane translocating sequence capable of locating a fusion protein on the outer surface

15   and a gene segment encoding any of a variety of proteins.

       Overall and in general the tripartite chimeric genes of the invention include at least three DNA segments. One segment is a targeting DNA sequence encoding a

20   polypeptide capable of targeting and anchoring the fusion polypeptide to a host cell outer membrane. Targeting sequences are well known and have been identified in several of membrane proteins including Lpp. Generally, as in the case of Lpp, the protein domains serving as

25   localization signals are relatively short. The Lpp targeting sequence includes the signal sequence and the first 9 amino acids of the mature protein. These amino acids are found at the amino terminus of Lpp. *E. coli* outer membrane lipoproteins from which targeting

30   sequences may be derived include *TraT, OsmB, NlpB* and *BlaZ*. Lipoprotein 1 from *Pseudomonas aeruginosa* or the PAl and PCN proteins from *Haemophilus influenza* as well as the 17 kDa lipoprotein from *Rickettsia rickettsii* and the H.8 protein from *Neisseria gonorrhea* and the like may

35   be used.

A second component of tripartite chimeric genes is a
DNA segment encoding a membrane-transversing amino acid
sequence. Transversing is intended to denote an amino
acid sequence capable of transporting a heterologous or
5   homologous polypeptide through the outer membrane. In
preferred embodiments, the membrane transversing sequence
will direct the fusion polypeptide to the external
surface. As with targeting DNA segments, transmembrane
segments are typically found in outer membrane proteins
10   of all species of gram-negative bacteria. Transmembrane
proteins, however, serve a different function from that
of targeting sequences and generally include amino acids
sequences longer than the polypeptide sequences effective
in targeting proteins to the bacterial outer membrane.
15   For example, amino acids 46-159 of the *E. coli* outer
membrane protein OmpA effectively localize a fused
polypeptide to the external surface of the outer membrane
when also fused to a membrane targeting sequence. These
surface exposed polypeptides are not limited to
20   relatively short amino acid sequences as when they are
incorporated into the loop regions of a complete
transmembrane lipoprotein. While the invention has been
demonstrated with a transmembrane directing protein
sequence from OmpA, other transmembrane directing
25   sequences from outer membrane proteins may be employed.

The third gene segment comprising the tripartite
chimeric gene fusion is a DNA segment that encodes any
one of a variety of desired polypeptides. This DNA
30   segment is positioned downstream from the DNA segment
encoding the transmembrane sequence. The tripartite
chimeric gene when provided with a functional promoter is
expressible in gram-negative host cells.

35   A particular embodiment of the invention includes
recombinant vectors prepar d from the her in-described
tripartite chimeric gene fusions. Such vectors will

express fusion polypeptides at the outer membrane cell
surface of a gram-negative host cell. These r combinant
vectors include a functional promoter sequence and a
targeting DNA sequence encoding a protein capable of
5   targeting to the outer surface of a gram-negative
bacterial host cell. The targeting gene is typically
positioned downstream of the promoter sequence. A
transmembrane gene sequence is positioned downstream of
the targeting gene sequence. The transmembrane sequence
10   will encode a protein domain capable of transversing the
cell outer membrane. The vector will also include a DNA
sequence which encodes a desired protein. This sequence
when positioned downstream of the transmembrane sequence
will be expressed on the external surface of the outer
15   membrane, and typically is exposed to the external medium
while remaining stably anchored to the membrane surface.

     A most preferred embodiment of the recombinant
vector is plasmid pTX101. This plasmid contains a fusion
20   of the signal sequence and the first 9 amino acids of the
major outer membrane lipoprotein of *E. coli*, a 342-base
pair fragment from the outer membrane protein OmpA and
the coding sequence for the complete mature β-lactamase
protein. However, clearly numerous variations of the
25   disclosed recombinant vectors could be prepared using
techniques well known to those of skill in the art. DNA
sequences encoding regions from a wide variety of
membrane proteins could be employed. Such regions may be
fused with any of a number of genes or gene fragments via
30   a polylinker region.

     The polypeptides encoded by the nucleic acid
segments identified herein have been described in terms
of function related to targeting and transversing fusion
35   polypeptides to a gram-negative bacterial cell uter
membrane surface. The inventi n is intended t include
variations of the fused genes disclosed to the extent

that the encoded polypeptides are functionally
biologically equivalent. In general, by biologically
functionally equivalent is meant amino acid sequences
that may vary from certain of the disclosed fusion
5   products, by e.g., natural genetic drift, strain or
subspecies antigenic variation or by mutation of the DNA
molecules without loss of appropriate membrane targeting
or transversing functions as described.

10   Likewise, certain changes in nucleic acid
composition of genes encoding polypeptides having the
aforementioned functions, will not affect the general
broad concept of the invention. For example, vectors
containing variant codons for a particular amino acid,
15   while altering the DNA composition, will not change the
amino acid identity. Minor base pair changes, while
producing some variation in primary amino acid sequence
of the encoded polypeptide, are not expected to
substantially alter function. All such variations,
20   whether in amino acid or nucleic acid composition, are
contemplated to be within the scope of the invention.

The methods illustrated for the expression of
desired recombinant polypeptides on the cell surface may
25   also be achieved by fusion to protein domains other than
those derived from the major lipoprotein and OmpA,
provided that these domains can function for the
expression of the desired polypeptide on the cell
surface. Generally, the desired polypeptide is fused to
30   an amino acid sequence that includes the signals for
localization to the outer membrane and for translocation
across the outer membrane. The amino acid sequences
responsible for localization and for translocation across
the outer membrane may be derived either from the same
35   bact rial protein or from different proteins of the same
or different ba terial species. Examples of proteins
that may serve as sources of localization signal domains

include *E. coli* outer membrane lipoproteins from such as
TraT, *OsmB*, *NlpB*, *BlaZ*, *Pseudomonas aeruginosa*
lipoprotein 1, *Haemophilus influenza* PA1 and PCN
proteins, *Rickettsia rickettsii* 17 kDa lipoprotein,

5 *Neisseria gonorrhea* H.8 protein and the like. A sequence
that spans the outer membrane and serves to transport the
desired recombinant polypeptide to the cell surface can
be derived from a membrane spanning domain of suitable
length from any native outer membrane protein of gram-

10 negative bacteria, including the porins LamB, PhoE, OmpC
and OmpF, as well as other outer membrane proteins such
as OmpT, FepA, and the like.

Any of a wide variety of gram-negative bacteria may

15 be useful in practicing the invention. Such gram-
negative bacteria include *E. coli*, *Salmonella*,
Klebsiella, *Erwinia*, and the like. *E. coli* and
Salmonella are particularly preferred as host cells.
Although there are variations among the bacteria outer

20 membrane proteins are similar. Target and transversing
sequences from any of the membrane proteins may be used
in constructing vectors useful for exportation across the
cell wall of gram-negative bacteria.

25 Another aspect of the invention includes
transformants. A typical transformant is a *Salmonella*
prepared by transformation with the described recombinant
vectors. A most preferred transformant is *E. coli*.

30 The invention is typically practiced using one or
more of the commonly available gram-negative bacteria as
cell hosts. However, rough mutants having somewhat
differing membrane compositions are expected to also be
useful in the practice of the invention. Membranes with

35 higher phospholipid content, for example, may for some
fusion polypeptides, provide mor efficient surfac
expression at higher temperatures. Alternativ ly, it may

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be desirable to anchor some polypeptides closer to the
membrane surface with increased lipid-protein
interactions, perhaps for the purpose of increasing
immunogenic response or altering adsorbent properties.
5   Such mutants, spontaneously generated or otherwise, are
contemplated as useful as host cells and/or as sources
for membrane directing and transversing sequences.

        Numerous types of fusion polypeptides may be
10   expressed using the aforementioned system. Relatively
large proteins such as alkaline phosphatase have been
expressed on the surface of *E. coli* host cells. In its
dimeric form alkaline phosphatase has a molecular weight
of greater than 80 kDa. Other large proteins are also
15   expected to be effectively surface expressed. Examples
of expressed polypeptides include ß-lactamase, alkaline
phosphatase, cellulose binding domain of cellulase, or
single-chain F_v antibody.

20   Expression of a variety of single-chain antibodies
on the surface of a gram-negative bacterial host cell has
several potential important applications particularly for
the preparation of whole cell adsorbents. In addition, a
variety of antigenic determinants may be expressed on a
25   cell surface and used to prepare bacterial vaccines. A
selected antigen in combination with an activating agent
such as IL-4 on the surface of a bacterium may have
potential use in stimulating an immune response toward a
surface exposed antigen.
30
        Tripartite chimeric gene fusions or the recombinant
vectors herein described will typically include
appropriate promoters. Such promoters are well known to
those of skill in the art and examples include *lpp*
35   promoter or *lac* promoter. Additionally, recombinant
vectors also include a signal peptide. In preferred

embodiments the signal peptide is positioned upstream of the targeting gene segment in recombinant vectors.

The invention also includes a method for expressing
5 a fusion polypeptide anchored on the outer membrane surface of a gram-negative bacterial host cell. A gene segment encoding a desired polypeptide is selected and inserted by the herein described methods into one or more of the disclosed recombinant vectors. A selected gram-
10 negative cell is transformed with the vector. The transformants are cultured and screened in order to identify clone transformants having a desired peptide expressed on the host cell surface. There are numerous ways the desired gene segment encoding the polypeptide
15 could be incorporated into one or more of the disclosed recombinant vectors. For example, plasmid pTX101 may be cut with the restriction endonuclease EcoRI at the unique site in the linker region between the OmpA and ß-lactamase sequence. Typically, blunt ends are created on
20 the DNA by treating with the Klenow fragment of DNA polymerase. Plasmids containing the coding sequence for the desired polypeptide may be isolated and DNA fragments obtained by cutting that plasmid with an appropriate endonuclease followed by blunt ending again using a
25 Klenow fragment or similar polymerase. The linearized pTX101 vector and the desired gene fragment may then be ligated and the resultant DNA transformed into an appropriate bacterial host cell strain such as E. coli strain JM109.

30

Surprisingly, the temperature at which the cells are cultured has an effect on the expression of the desired polypeptide. Culturing at higher temperatures, about 40°C, for example, results in less efficient expression
35 of the desired polypeptide on the surface of the bacterial host. Although expression on th surface may b obtained when culturing is performed between about

22°-40°C, a preferred temperature range is between 22°-27°C and a most preferred temperature being around 24°C.

Yet another aspect of the present invention is a
5   method for obtaining an immunogenic polypeptide. An
immunogenic polypeptide to which it is desired to elicit
an immune response is selected and then inserted into an
appropriate recombinant vector prepared in accordance
with the aforementioned procedures. Appropriate gram-
10 negative cells are transformed and the culture screened
for transformants. Transformants are then screened to
determine the degree of immunogenicity and those that are
highly immunogenic are used to obtain one or more
antibodies. This method is particularly useful because
15 it is known that surface expressed polypeptides typically
elicit higher antigenic and immunogenic responses than
those peptides that are not immobilized on a bacterial
surface. Surface exposed immunogenic polypeptides may
also be used to prepare vaccines, typically by mixing the
20 cells in a pharmaceutically acceptable vehicle suitable
for administration in mammals.

Antibodies can be equally well expressed on the
surface of the cell. When such antibodies are expressed
25 on cell surfaces those with high affinity for particular
antigens may be selected. Variants of antibodies may be
prepared and surface expressed and antibody-like
sequences may be prepared and tested for affinity to the
appropriate antigens.
30
In yet another aspect of the invention it is
contemplated that kits useful for transforming gram-
negative bacterial host cells may be prepared. Kits will
include at least one recombinant vector prepared in
35 accordanc with the herein described invention in an
appropriately compartmentalized container. A preferred
recombinant vector is defined by SEQ ID NO:1.

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The invention also includes a method for removing
contaminants from fluids. In this method various
receptor proteins expressed on the outer membranes of
gram-negative bacteria may be used to selectively
5   interact with a wide variety of undesirable compounds.
Metallothionein, for example, binds with a wide variety
of heavy metals including iron, cadmium, zinc, copper,
vanadium, and similar metals. When bound to the surface
of a gram-negative organism this protein is expected to
10   efficiently remove heavy metals from aqueous samples.

Whole cell adsorbents, with surface expressed
polypeptides, such as selected antibodies, may be used to
remove biological contaminants, for example, bacterial
endotoxin from water samples. The efficiency of such
15   whole cell adsorbents may be increased by cross-linking
the bacterial surface. This also may increase the
stabilization of the cells against disruption. One
method of stabilization involves the specific cross-
linking of the cells through the lipopolysaccharide
20   component of the surface. Thus the cells can be
aggregated and stabilized without affecting the function
of surface-expressed proteins. Other types of cell
adsorbents are contemplated including the use of
cellulose binding domains, starch binding domains,
25   protein A, lectins, or protease receptors expressed on
outer membrane bacterial cell surfaces.

Still further embodiments of the invention include
30   immobilized enzyme systems. Any one of a wide variety of
biocatalytically active polypeptides may be expressed on
the surface of a bacterial cell using the disclosed
methods. Advantages of having an enzyme expressed on the
bacterial cell surface include increased accessibility to
35   substrat s, stability, and potentially increased lipid
solubility. In a mor particular embodiment,
biocatalytically active polypeptides immobilized on host

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cell membranes without additional bacterial host cell
components may be used in biphasic reacti n systems.
Enhanced lipid solubility of the immobilized enzymes
enables catalyst substrate interaction in the lipophilic
5   solvents with extraction of the water soluble products
into the aqueous phase. Further contemplated embodiments
in such an immobilized system include encapsulating
immobilized enzymes on membrane surfaces within liposomes
or similar vesicles.
10

As part of the invention, kits useful for the
expression of fusion proteins are also envisioned
comprising a container having suitably aliquoted reagents
for performing the foregoing methods. For example, the
15   containers may include one or more vectors, examples
being the vectors of claim 2, particular embodiments of
which are shown schematically in Figure 5. Suitable
containers might be vials made of plastic or glass,
various tubes such as test tubes, metal cylinders,
20   ceramic cups or the like. Containers may be prepared
with a wide range of suitable aliquots, depending on
applications and on the scale of the preparation.
Generally this will be an amount that is conveniently
handled so as to minimize handling and subsequent
25   volumetric manipulations. Most practitioners will prefer
to select suitable nucleases such as EcoRI, BamHI, or
PstI from common supplies usually on hand; however, such
restriction endonucleases could also be optionally
included in a kit preparation.
30

Vectors supplied in kit form are preferably supplied
in lyophilized form, although such DNA fragments may also
be taken up in a suitable solvent such as ethanol,
glycols or the like and supplied as suspensions. For
35   most applications, it would be desirable to remove the
solvent which for ethanol, for example, is a relatively
simple matter of evaporation.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the Lpp-OmpA-ß-
lactamase fusion in the outer membrane of a gram negative
5   bacterium. Rectangles represent membrane-spanning ß-
strands of OmpA.

Figure 2 shows the fractionation of membranes from
JM109(pTX101) and JM109 cells. The percent of total
10   membrane protein and ß-lactamase activity in different
fractions from a sucrose gradient of pTX101 is shown. ß-
lactamase activity was determined from the rate of
hydrolysis of penicillin G. Fractions 2-7 had an average
density of 1.22 g/cc, 11-13: p=1.19 g/cc and 16-20:
15   p=1.15g/cc corresponding exactly to the values for outer
membrane, intermediate and inner membrane vesicles
determined by Osborn.

Figure 3 shows fractionation on a sucrose gradient
20   of membranes from JM109(pTX101) and JM109(pJG311).
Figure 3A: Samples from every three fractions were
pooled together and loaded in consecutive lanes. Lanes
2-8, pTX109: lanes 9-15, pJG311. Lanes: 1: Molecular
weight markers: 2: fractions 1-3; 3: fractions 4-6; 4:
25   fractions 7-9; 5: fractions 10-12; 6: fractions 13-15; 7:
fractions 16-18; 8: fractions 19-21; 9: fractions 1-3;
10: fractions 4-6;11: fractions 7-9; 12: fractions 10-12;
13: fractions 13-15; 14: fractions 16-18; 15: fractions
19-21; 16: molecular weight markers. The molecular
30   weight standards (BRL) are: myosin H-chain, 200 kDa:
phosphorylase B, 97 kDa: bovine serum albumin, 68 kDa:
ovalbumin, 43 kDa: and carbonic anhydrase, 29 kDa.
Arrows indicate the fusion proteins Lpp-OmpA-ß-lactamase
(lane 2) and Lpp-ß-lactamase (lane 9). Figure 3B:
35   Western blot of the JM109(pTX101) fractions fr m the
sucrose gradient (Figure 3A, lanes 2-8). The primary
antibody was used at a concentration of 1:20.000. The

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gel was overloaded to show the presence of degradation
products. There were no degradation products below the
32.000 dalton molecular weight standard. As with the
native ß-lactamase, the Lpp-OmpA-ß-lactamase migrates as
5   two bands depending on the oxidation of the single
disulfide bond (30). The prestained molecular weight
markers (Bio-Rad) have apparent molecular weights of: 106
kDa, phosphorylase B: 80 kDa, bovine serum albumin: 49
kDa, ovalbumin: 32 kDa, bovine carbonic anhydrase.
10

Figure 4 shows micrographs of the same field of
JM109(pTX101) cells labelled with rabbit ß-lactamase
specific antibodies and rhodamine conjugated rabbit-
specific antibodies viewed by fluorescence (4A) and phase
15   contrast microscopy (4B).

Figure 5 shows the effect of extended incubation on
measured ß-lactamase activity of the tripartite fusion
expressed on the outer membrane surface of *E. coli*.
20

Figure 6 shows a diagram of the plasmid pTX101.

Figure 7 (SEQ ID NO:1) shows the DNA sequence of the
genes coding for the tripartite fusion from pTX101.
25

Figure 8 shows the condons for the segments included
in the tripartite fusion from pTX101.

Figure 9 shows a scanning electron micrograph of
30   JM109(pTX101) cells labelled with anti-ß-lactamase
antibodies and secondary gold conjugated antibodies.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Materials and Methods

5   Bacterial Strains

E. coli strain JM109 (*endA1 recA1 gyrA thi-1
hsdR17*(r_k^-, m_k^+) *relA1 supE44* X(*lac-proAB*)/F' *traD36 proAB
lacI*^{q} *lacZΔM15*) (Invitrogen, San Diego, CA), ATCC 53323.

·10
Escherichia coli strain JCB572 is obtained from Dr.
J. Beckwith, Department of Molecular Biology and
Molecular Genetics, Harvard Medical School, Boston, MA
02115 (Bardwell *et al.*, 1991).

15
Plasmids

Plasmid pSWFII is prepared as described by Ehrmann
et al., 1990.

20
Plasmid pJG311 (Ghrayeb and Inouye, 1984; Yamaguchi
et al., 1988) was constructed by cutting pMH014 Cm^r
(Yamaguchi *et al.*, 1988) which contains the gene coding
for the signal sequence and mature major outer membrane
25   lipoprotein, with *EcoRI* and then removing all the
lipoprotein gene except the signal sequence and the first
nine amino acids. The β-lactamase gene, cut from pTG206,
was ligated into this site creating pJG202. The region
coding for the lipoprotein signal sequence, the first
30   nine amino acids from the lipoprotein, and the entire
mature β-lactamase, was transferred from pJG202 to the
expression plasmid pIN-III-A-Cm^r (Yoshihiro *et al.*, 1983)
to create pJG311, which contains a Lpp-β-lactamase
fusion. Plasmid pJG311 may also be obtained from
35   Masayori Inouye, Department of Biochemistry, Robert Wood
Johnson Medical at Rutgers University of Medicine and

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Dentistry of New Jersey, Piscataway, New Jersey USA
08854.

Plasmid pRD87 is constructed in the same manner as
5   pTU500 (Freudl et al., 1985). pTU500 is constructed by
cutting the OmpA gene from pTU500/1 and ligating it into
pUC9 (Vieira and Messing, 1982) thereby placing it under
the control of the lac promoter. pRD87 was made
identically except that the ompA gene from pTU500/1 was
10   cloned into pUC8 (Vieira and Messing, 1982). The two
plasmids, pTU500 and pRD87, are identical except that
pTU500 contains an amber mutation at the seventh codon in
the ompA sequence, while pRD87 does not contain the amber
mutation. Plasmid pRD87 may also be obtained from Ulf
15   Henning, Max-Planck-Institut für Biologie, Corrensstrasse
38, D-7400 Tübingen, Germany.

Cultures

20   Cultures were grown in either LB medium (Difco)
supplemented with 0.2% glucose or M9 medium supplemented
with 0.2% casein amino acid hydrolysate and 0.2% glucose.
The desired antibiotics were added as required.

25   General Procedures

SDS-PAGE was performed on 11% and 15% acrylamide
gels. Protein samples, denatured for 5 minutes in boiling
SDS containing ß-mercaptoethanol, were loaded onto
30   polyacrylamide gels and run at a constant current. The
gels were stained with Coomassie brilliant blue (R 250)
for 15 minutes and the background stain was removed
overnight with a methanol/acetic acid destaining
solution.
35

Western blots were performed by running 0.5 μg
pr tein samples on polyacrylamide gels at constant

current and were transferred overnight to nitrocellulose membranes. The membranes were incubated for 1 hour with rabbit anti-ß-lactamase antibodies, rinsed, and incubated for 1 hour with horseradish peroxidase conjugated goat

5   anti-rabbit antibodies. After further rinsing, the membranes were developed with 4-chloro-1-naphthol, which gives a distinct blue color at the sites containing horseradish peroxidase.

10   The enzymatic activity of ß-lactamase was measured by the rate of hydrolysis of penicillin G or nitrocefin (Samuni, 1975; O'Callaghan et al., 1972). Hydrolysis of penicillin G gives linear decrease in the adsorption of light at 240 nm, while nitrocefin hydrolysis shows an

15   adsorption increase at 482 nm. The changes in adsorption with time were measured in an LKB spectrophotometer. Protein concentrations were measured by the Bio-Rad assay using standard curves prepared from protein standards and comparing color developed with the reagent measured at

20   595 nm in a spectrophotometer.

The present invention relates to a novel chimeric gene from which a wide variety of recombinant expression vectors useful for surface expression of desired proteins

25   is possible. Appropriately transformed gram-negative host cells will efficiently express proteins on the outer membrane surface without loss of inherent activity. A novel aspect of the fused gene is the use of three separate DNA segments which act respectively (1) to

30   target a fusion product to the host cell outer membrane and (2) to transverse the fusion product across the membrane to the outer surface where (3) the polypeptide expressed by a fused gene of interest becomes stably anchored to the surface.

35

The particular examples herein illustrated utilize recombinant vectors constructed from known DNA segments

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having particular functions. For example, various
membrane proteins such as OmpA are known to contain both
membrane targeting and transversing sequences. However,
fusion of alkaline phosphatase with outer membrane

5   proteins has not produced surface expressed alkaline
phosphatase (Murphy et al., 1990). The present invention
utilizes separate targeting and transversing domains.
When engineered into a vector such that a gene for a
desired polypeptide product is positioned downstream of

10   both the targeting and transversing sequences, efficient
surface expression of the product is effected. Moreover,
the targeting sequence is positioned upstream of the
transversing sequence.

15   It will be appreciated that the particular gene
sequences shown here to construct a tripartite chimeric
gene are not limited to deriving targeting and
transversing sequences from lipoprotein and OmpA
respectively. Other sequences with analogous function

20   may be used. In particular, this invention may be
efficiently practiced with the construct shown
schematically in Figure 6 and in particular detail in
Figure 7 (SEQ ID NO:1) illustrates useful targeting and
transversing DNA sequences fused with the ß-lactamase

25   gene, although it is appreciated that numerous other
polypeptide sequences could be used rather than ß-
lactamase.

        The invention has numerous applications, a brief
30   background for which is described.

Whole Cell Affinity Adsorbents

        Affinity purifications of biomolecules rely
35   primarily on the strong interactions between proteins and
ligands. Typically, the ligand is bound to a solid
support matrix which is employed in a chromatographic-

type separation. More rec ntly, suspensions of starch
granules (Mattiasson and Ling 1986) or liposomes (Powell
et al. 1989) have been used as supports for affinity
purifications. In some of the most useful and specific
5   separations, the affinity ligands are proteins such as
antibodies, lectins or protein receptors (Mohr and
Pommerening 1986, Turkova 1978). The preparation of
protein affinity adsorbents involves the production,
purification and the immobilization of the polypeptide on
10   a solid support matrix. These three steps are generally
complicated and often prohibitively expensive for large
scale applications. On the other hand bacterial cells
expressing proteins on their surface can serve as an
important source of low cost solid phase adsorbents.

15
        The human metallothionein gene protein has been
expressed as a fusion with an outer membrane protein
(Jacobs et al., 1989). Because of the way the fusion
protein was constructed, metallothionein was localized on
20   the internal side of the E. coli outer membrane, i.e.,
facing the periplasmic space. Nevertheless, since metal
ions can diffuse through the outer membrane, the
recombinant cells were able to bind as much as 66 fold
more Cd^{+2} than normal E. coli. Another example of a high
25   affinity cellular adsorbents (e.g., Kronvall et al. 1979)
includes the use of cultured mammalian cells to remove
viral impurities from blood samples (Tsao et al., 1988).

Whole Cells As Enzyme Carriers For Bioprocessing

30
        The use of whole cells as enzymatic catalysts has
been in use for several years. Typically, a
microorganism which produces a certain enzyme is used as
a biocatalyst, thus avoiding the costs associated with
35   protein purification and immobilization steps. Usually
th c lls are first killed, tr ated with a permeabilizing
agent to allow the diffusion of reactants and products

into the cytoplasm and finally they are stabilized using
some form of chemical crosslinking (Tampion and Tampion
1987). Several improvements on the preparation of whole
cell biocatalyst have been made over the years. However,

5   certain inherent limitations can not be overcome with the
currently available technology. These are: i) The
chemical methods which are used for permeabilization of
the cell membrane can also result in deactivation of the
important enzyme; ii) Other intracellular enzymes may

10   compete for the reaction substrate giving rise to
undesired byproducts and decreased yields; and iii)
Intracellular degradation processes can limit the
functional life of the biocatalyst. Clearly, all these
problems can be eliminated if the enzyme is attached to

15   the cell's exterior.

Live Bacterial Vaccines

Genetically weakened (attenuated) strains of

20   bacteria that are able to survive and persist in the
human or animal body can confer prolonged immunological
protection against disease (Stover 1991). Non-
recombinant live vaccines have been used for many years
for large scale vaccinations (Dougan 1989). For example,

25   live attenuated cultures of Baccillus Calmette-Guérin
(BCG) which confer immunity against tuberculosis
represent the most widely used vaccine in the world
(Stover et al. 1991). Recently, emphasis has been
shifted to the development of recombinant bacterial

30   vaccines (Curtiss et al. 1989, Charles and Dougan 1990).
In this case vaccination consists of the oral
administration of a live culture of an attenuated enteric
bacterium host such as E. coli or Salmonella typhimurium
which expresses an antigenic peptide from a pathogen.

35   Within the body, some of the bacteria find their way to
the intestinal tract where they coexist with the wild
type E. coli and other enteric microorganisms. In this

way they ensure the presence of a l w level of antigenic
peptide in the body. Live vaccines provide more
efficient immunity and longer protection against
infections compared to subunit or killed bacterial
5   vaccines. There are several reasons for the higher
efficacy of live bacterial vaccines (Dougan et al. 1989):
i) Protection correlates with how long the vaccine is
present in the body (De Libero and Kaufman, 1986). Since
the bacteria persist in the intestine for very long
10   times, they are able to confer extended immunity; ii)
Unlike most currently used vaccines, bacterial vaccines
may be administered orally; and iii) Several antigens may
be expressed simultaneously in bacteria thus giving rise
to multipurpose vaccines.

15
     Although the foregoing antigen may stimulate an
immune response even when produced within the cell, the
immunogenicity of peptide antigens can be greatly
enhanced if they are expressed on the surface of an
20   appropriate host strain (Taylor et al. 1990). This is
because the surface of the bacteria such as Salmonella or
E. coli acts as an adjuvant to enhance the immune
response to the antigen. The most straightforward way to
accomplish this is to insert the foreign peptide within a
25   surface exposed loop of an outer membrane protein which
serves as the targeting signal. A fusion protein with
the structure outer membrane protein--peptide--outer
membrane protein is constructed and then the normal
protein localization mechanism of the cell is exploited
30   to carry the peptide to the surface. There appears,
however to be an upper limit on the length of foreign
polypeptides that can be inserted within outer membrane
proteins. The maximum size of foreign sequence that can
be accommodated within outer membrane proteins is around
35   45 to 50 amino acids (Newton et al. 1989, Charbit et al.
1988).

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Several different outer membrane proteins have been
exploited as targeting vehicles for the localization of
foreign peptides (e.g., Charbit et al., 1988). A number
of short amino acid sequences have been inserted within a
5   surface exposed loop of the E. coli outer membrane
protein maltoporin (LamB) (Charbit et al., 1988). The
peptides were localized correctly so that the whole cells
could be used to induce an immune response. Expression
systems for the localization of antigenic peptides on
10   bacterial surfaces have also been constructed using the
E. coli K88ac and K88ad pilin proteins (Thiry et al.
1989), the S. typhimurium flagellin, (Newton et al. 1989)
the TraT lipoprotein (Taylor et al. 1990) and the E. coli
outer membrane porins PhoE, OmpA and OmpC (Agterberg
15   1987, Freundl 1989).

Prototype live bacterial vaccines have been prepared
using cells having sequences from the influenza virus,
cholera toxin B subunit and the gp 120 glycoprotein of
20   HIV-1 expressed on their surface. However, the presence
of a fragment of a protein from an infectious agent often
does not give satisfactory protection against disease
(Dougan et al. 1989).

25   An advantage of the present invention is the
potential to express complete proteins from infectious
agents on the surface of the carrier cells. Immunization
with an intact protein is more likely to elicit a humoral
immune response and provide protective immunity.
30

Vaccine Preparation and Use

Preparation of vaccines which contain peptide
sequences as active ingredients is generally well
35   understood in the art, as exemplified by U.S. Patents
4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792;
and 4,578,770, all incorporated herein by reference.

Typically, such vaccines are prepared as injectabl s
either as liquid solutions or suspensions; solid forms
suitable for solution in, or suspension in, liquid prior
to injection may also be prepared. The active
5   immunogenic ingredient is often mixed with excipients
which are pharmaceutically acceptable and compatible with
the active ingredient. Suitable excipients are, for
example, water, saline, dextrose, glycerol, ethanol, or
the like, and combinations thereof. In addition, if '
10   desired, the vaccine may contain minor amounts of
auxiliary substances such as wetting or emulsifying
agents, pH buffering agents, or adjuvants which enhance
the effectiveness of the vaccines.

15   Live bacterial vaccines are conventionally
administered parenterally, by injection or in oral
formulation. Oral formulations include such normally
employed excipients as, for example, pharmaceutical
grades of mannitol, lactose, starch, magnesium stearate,
20   sodium saccharine, cellulose, magnesium carbonate, and
the like. These compositions take the form of solutions,
suspensions, tablets, pills, capsules, sustained release
formulations or powders and contain 10-95% of active
ingredient, preferably 25-70%.   '

25
          The vaccines are administered in a manner compatible
with the dosage formulation, and in such amount as will
be therapeutically effective and immunogenic. The
quantity to be administered depends on the subject to be
30   treated, including, e.g., the capacity of the
individual's immune system to synthesize antibodies, and
the degree of protection desired. Precise amounts of
active ingredient required to be administered depend on
the judgment of the practitioner. However, suitable
35   dosage ranges are of the order of several hundred
micrograms active ingredient per vaccination. Suitable
regimes for initial administration and booster shots are

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also variable but are typified by an initial
administration followed by subsequent inoculations or
other administrations.

5   The manner of application may be varied widely. Any
of the conventional methods for administration of a
vaccine are applicable. These are believed to include
oral application on a solid physiologically acceptable
base or in a physiologically acceptable dispersion,
10   parenterally, by injection or the like. The dosage of
the vaccine will depend on the route of administration
and will vary according to the size of the host.

         In many instances, it will be desirable to have
15   multiple administrations of the vaccine, usually not
exceeding six vaccinations, more usually not exceeding
four vaccinations and preferably one or more, usually at
least about three vaccinations. The vaccinations will
normally be from two to twelve week intervals, more
20   usually from three to five week intervals. The course of
the immunization may be followed by assays for antibodies
for the supernatant antigens. The assays may be
performed by labeling with conventional labels, such as
radionuclides, enzymes, fluorescers, and the like. These
25   techniques are well known and may be found in a wide
variety of patents, such as U.S. Patent Nos. 3,791,932;
4,174,384 and 3,949,064, as illustrative of these types
of assays.

30   The invention also contemplates the use of disclosed
nucleic acid segments in the construction of expression
vectors or plasmids and use in host cells. The following
is a general discussion relating to such use and the
particular considerations in practicing this aspect of
35   the invention.

Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for
the initial cloning of DNA sequences and constructing the
vectors useful in the invention. For example, in
5   addition to the particular strains mentioned in the more
specific disclosure below, one may mention by way of
example, strains such as *E. coli* K12 strain 294 (ATCC No.
31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537).
10   These examples are, of course, intended to be
illustrative rather than limiting.

Prokaryotes are also preferred for expression. The
aforementioned strains, as well as *E. coli* W3110 (F-,
15   lambda-, prototrophic, ATCC No. 273325) or other
enterobacteriaceae such as *Salmonella typhimurium* or
Serratia marcesans, and various *Pseudomonas* species may
be used.

20   In general, plasmid vectors containing replicon and
control sequences which are derived from species
compatible with the host cell are used in connection with
these hosts. The vector ordinarily carries a replication
site, as well as marking sequences which are capable of
25   providing phenotypic selection in transformed cells. For
example, *E. coli* is typically transformed using pBR322, a
plasmid derived from an *E. coli* species (see, e.g.,
Bolivar et al., 1977). The pBR322 plasmid contains genes
for ampicillin and tetracycline resistance and thus
30   provides easy means for identifying transformed cells.
The pBR plasmid, or other microbial plasmid or phage must
also contain, or be modified to contain, promoters which
can be used by the microorganism for expression.

35   Those promoters most commonly used in recombinant
DNA construction include the lactose promoter systems
(Chang t al., 1978; Itakura et al., 1977; Goeddel et

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al., 1979) and a tryptophan (trp) promoter system
(Goeddel et al., 1979; EPO Appl. Publ. No. 0036776).
While these are the most commonly used, other microbial
promoters have been discovered and utilized, and details
5   concerning their nucleotide sequences have been
published, enabling a skilled worker to ligate them
functionally with plasmid vectors (Siebwenlist et al.,
1980).   Certain genes from prokaryotes may be expressed
efficiently in E. coli from their own promoter sequences,
10   precluding the need for addition of another promoter by
artificial means.

     The following examples are intended to illustrate
the practice of the present invention and are not
15   intended to be limiting. Although the invention is here
demonstrated with ß-lactamase expressed on the surface of
a cell membrane, numerous other proteins with various
functions could be similarly expressed. These would
include polypeptides with catalytic functions, metal
20   binding capability and specific binding activity toward
cell receptor sites. Moreover, the expression vectors
and chimeric genes used therein may be constructed from a
wide variety of targeting and transversing sequences, and
are not limited to those derived from OmpA and Lpp.

25

                          EXAMPLE 1

     The following example illustrates the construction
30   of a recombinant vector encoding a desired protein,
targeting and membrane translocating sequences. When
used to transform suitable bacterial hosts, such a vector
enables surface expression of active proteins, as shown
here for the production of ß-lactamase.

35

Construction of Plasmid pTX101 Containing Tripartite Chimeric Gene for β-Lactamase Expression

Plasmid pTX101 was prepared from pJG311, which
5   contains the signal sequence and first 9 N-terminal amino
acids of the mature major outer membrane lipoprotein of
E. coli and the complete β-lactamase sequence. A unique
EcoRI site in the linker region between the peptide
region and the β-lactamase was cut with EcoRI. The cut
10 plasmid was isolated from a low melting point agarose gel
and the ends were made blunt using the Klenow fragment.
pRD87, containing the OmpA gene, was simultaneously cut
with HpaI and SphI, both unique sites, generating a 342
bp fragment containing the sequence for five of the eight
15 outer membrane spanning domains of OmpA. This fragment
was isolated from a low melting point agarose gel and
made blunt with T4 DNA polymerase. The fragment, coding
for amino acid residues 46-159 of OmpA, was ligated to
the above pJG311 vector to make pTX101, which codes for
20 the Lpp-OmpA-β-lactamase fusion. The ligation was
transformed into E. coli strain JM109 made competent by
the rubidium chloride method.

The Lpp-OmpA-β-lactamase was expressed from the
25 strong lpp promoter which is inducible by IPTG (isopropyl
thiogalactoside). Plasmid pTX101 also carries the lacI
repressor. Although induction with IPTG resulted in high
levels of protein production which are lethal to the
cell, good expression was nevertheless obtained in the
30 absence of inducer. Cultures were harvested in late
exponential phase and the cells lysed and separated into
soluble and cell envelope fractions by high speed
centrifugation. Approximately 84% of total β-lactamase
activity from JM109(pTX101) lysates was found in the cell
35 envelope fraction. Essentially all the remaining
activity was present in the soluble fraction of the cell
lysates with less than 0.5% in the extracellular fluid.

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Even after prolonged incubation of stationary phase cells
(24 hrs) there was no increase in the percentage of ß-
lactamase in the extracellular fluid, indicating that the
fusion protein was not released from the cells, Figure 5.

5   Qualitatively similar results were observed when the
distribution of the fusion protein in the different
fractions was examined by immunoblotting with ß-lactamase
or OmpA-specific antisera. The Lpp-ß-lactamase protein
from plasmid pJG311 had 2-fold higher total activities

10 compared to the three-part fusion which contained the
OmpA insert. This protein was also found predominantly
in the membrane pellet (98% of total activity).

                            EXAMPLE 2

15

     This example illustrates that a heterologous
polypeptide prepared as in Example 1 is exposed to the
external medium and retains activity.

20 **Cell Fractionation**

     First it was demonstrated that the fusion protein
was localized to the outer membrane. Cells were
harvested from 200 ml of LB medium containing 0.2%

25 glucose at an $A_{600}=1.0$, washed in 25 mM Tris-HCl (pH 7.4)
and resuspended in 10 ml of the same buffer containing 1
mM EDTA and 100 µg/ml lysozyme at 4°C. After a 2 minute
incubation the cells were lysed by two passages through a
French pressure cell at 10,000 psi. The cellular debris

30 was removed by centrifugation at 2,500 xg for 8 minutes
and the total membranes were spun down by centrifugation
at 115,000 xg for 1 hour. Membranes were resuspended in
0.8 ml of Tris-HCl buffer containing 25% sucrose and
loaded onto a step gradient of 20, 35, 40, 45, 50 and 55%

35 (w/w) sucrose. After centrifugation at 165,000 xg for 16
hours in a Beckman SW41Ti rotor, 0.5 ml fractions were
collected from the bottom of the tube. The density of

the fractions was determined from r fractive index
measurements. The concentration of sucrose was lowered
to <10% (w/w) by diluting the samples with Tris-HCl
buffer followed by centrifugation to pellet the

5 membranes.

Two distinct protein peaks were obtained in
fractions having the expected densities for inner and
outer membrane vesicles (Osborn et al., 1972). Virtually

10 all the ß-lactamase enzymatic activity was found in the
higher density fractions which corresponded to the outer
membrane vesicles, Figure 2. A protein band migrating at
a molecular weight of approximately 43,000 daltons, the
expected size of Lpp-OmpA-ß-lactamase band was found and

15 it was comparable in abundance to that of the major outer
membrane proteins, Figure 3A. The fusion protein was
subjected to some degradation resulting in the appearance
of lower molecular weight bands that crossreacted with ß-
lactamase-specific antibodies in immunoblots, Figure 3B.

20 The relatively small proportion of degradation fragments
indicated that most of the fusion protein was not
subjected to proteolysis.

Exposure of ß-Lactamase on Cell Surface

25

Localization of the ß-lactamase domain with respect
to the external surface of E. coli was determined by
various immunocytochemistry methods, activity assays and
protease accessibility experiments. For

30 immunofluorescence determinations, whole cells were
labelled with rabbit ß-lactamase-specific antibodies
followed by secondary rhodamine-conjugated goat anti-
rabbit antibodies. Washed, mid-exponential phase cells
were resuspended in phosphate buffered saline (PBS) with

35 or without 0.1 mg/ml trypsin and incubated at 37°C.
Soyb an trypsin inhibitor was added at different times to
stop the reaction and incubation at 37°C was continued

for a total of 1 hour. All subsequent procedures were
conducted at room temperature. The cells were washed
with PBS, incubated for 45 minutes with PBS and 1% bovine
serum albumin, washed and then incubated in the same

5   PBS/BSA solution with rabbit anti-β-lactamase antibodies
at 1:1,000 dilution for 45 minutes. Following another
three washes with PBS/BSA, the cells were mixed with
rhodamine-conjugated goat anti-rabbit antibodies,
incubated for 45 minutes and then washed three more

10   times. Finally, the cells were resuspended in PBS and
examined by phase contrast and video enhanced
fluorescence microscope.

In control experiments, no fluorescence above

15   background was detectable with JM109(pJG311) cells
expressing the Lpp-β-lactamase fusion protein, indicating
that there was no exportation to the outer surface.
Figures 4A and 4B show a comparison of the same field of
JM109(pTX101) cells viewed with fluorescence phase

20   contrast microscopies. Nearly all the cells became
fluorescent, indicating sequences recognized by the anti-
β-lactamase antibodies. Incubation with trypsin for
various times prior to antibody labelling resulted in a
gradual decrease in the fluorescent signal. After 1 hour

25   of incubation no signal was detected.

For immunoelectron microscopy cells were labelled
with rabbit anti-β-lactamase specific antibodies, washed
in various buffers as described above for the

30   immunofluorescence experiments and reacted with secondary
30 nm diameter colloidal gold conjugated goat anti-rabbit
antibodies. The labelled cells were positively stained
with uranyl-acetate and viewed by scanning electron
microscopy. In control experiments, no labelling

35   occurred with JM109(pJG311) cells. Figure 9 shows
JM109(pTX101) cells so labelled, indicating the presence

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of sequ nces recognized by the anti-ß-lactamase
antibodies on the external surface.

     The presence of enzymatically active ß-lactamase on
5   the cell surface was determined from protease
accessibility experiments and the rates of hydrolysis of
substrates not readily diffusible through the outer
membrane. Cultures grown in M9 medium were harvested at
A_{600}=1.0, washed with fresh medium and resuspended in M9
10 salts without glucose or antibiotics. The ß-lactamase
activity in the whole cells was determined using
nitrocefin and penicillin G as substrates. The cells
were incubated for 1 hr at 37°C in the presence or
absence of 0.1 mg/ml of either proteinase K or trypsin.
15 The protease digestions were stopped by adding 10 mM
phenylmethylsulfonyl fluoride or 0.2 mg/ml soybean
trypsin inhibitor respectively. Subsequently, the cells
were lysed and centrifuged at 2500 xg for 8 min to remove
unbroken cells. The membranes were pelleted as described
20 above, resuspended in 50 mM potassium phosphate buffer,
pH 6.5 and the remaining enzymatic activity measured.

     In cells containing plasmid pTX101, approximately
20% of the total membrane-bound activity was reproducibly
25 lost after a one hour incubation with either trypsin or
proteinase K, compared with only a 3% decrease in
JM109(pJG311), Table 1. A comparable, somewhat higher
percentage of surface exposed activity was obtained from
the rates of hydrolysis of nitrocefin in intact and lysed
30 cells. Nitrocefin does not cross the outer membrane of
E. coli and therefore can be used to test activity of
extracellular ß-lactamase (Kornacker and Pugsley, 1990).
The rate of hydrolysis of nitrocefin by intact cultures
of JM109(pJG311) was in agreement with results of
35 prot ase accessibility studies, indicating Lpp-ß-
lactamase is not transported across the cell surface.
Approximately 20-30% of the enzymatic activity f Lpp-

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OmpA-ß-lactamase was surface exposed on cells grown at 37°C, a significant increase over the background in control cultures, see Table 1.

TABLE 1

Percent Surface Exposed ß-Lactamase as Determined by Protease Accessibility and Enzymatic Activity Using Nitrocefin

Plasmid	Temperature	Percent decrease in penicillin G hydrolysis following incubation with:		Nitocefin activity in intact cells as percentage of total activity in membranes[b]
		Proteinase K[a]	Trypsin[a,b]	
pJG311	37°C	3	3	6
pTX101	37°C	23	18	38
pTX101	24°C		89	81

[a]   Cells were incubated with proteinase K or trypsin for one hour and the total membrane fractions were isolated as described in the materials and methods sections. The percent of exposed ß-lactamase corresponds to the activity remaining after incubation with proteases relative to untreated cells.

[b]   The standard deviation for all experiments was less than ±5% of the reported mean values.

EXAMPLE 3

The following example demonstrates that even after extended incubation, the region of the tripartite fusion containing the target protein remains stably anchored to the outer membran f the host cell. This example demonstrates the surface stability of surface-expressed fusion polypeptides using the disclosed methods.

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Surface Stability of Translocated Fusion Protein β-Lactamase

JM109 cells with the plasmid pTX101 were grown in LB
5   supplemented with glucose and ampicillin. After 4, 6, 8
and 24 hours, 10 ml samples were collected and separated
into culture supernatant, soluble and membrane fractions,
Figure 5. The cells were first pelleted by
centrifugation at 8,000 xg and the resulting supernatant
10   was saved as the culture supernatant fraction. The
pelleted cells were resuspended in 50 mM potassium
phosphate buffer (pH 7) and lysed in a French pressure
cell at 20,000 psi. The lysed samples were centrifuged
at 2,500 xg to pellet any unlysed cells and then
15   centrifuged for 1 hr at 115,000 xg. The supernatant from
the high speed centrifugation was removed and saved as
the soluble fraction and the pelleted membranes
resuspended in 50 mM potassium phosphate buffer to obtain
the membrane fractions. β-lactamase activity performed
20   on the fractions indicated that even after prolonged
incubation (24 hrs) the fusion was stably anchored to the
outer membrane (Figure 5).

EXAMPLE 4

25
This example illustrates that efficient surface-
expression and maintenance of activity of surface-
expressed polypeptides is affected by the culture
temperature. The example is illustrated with β-
30   lactamase, but effective surface expression with
maintenance of function is also affected by temperature
for alkaline phosphatase.

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<u>Effect of Temperature on β-Lactamase Expression and</u>
<u>Activity</u>

5   Cultures grown at 24°C exhibited almost quantitative
β-lactamase activity on the cell surface. The rate of
nitrocefin hydrolysis and trypsin accessibility indicated
80-87% surface exposure, Table 1.

EXAMPLE 5

10

        The following example illustrates expression of
alkaline phosphatase on the outer membrane surface of *E.*
coli. Alkaline phosphatase is a large dimeric enzyme
with a monomer size of approximately 43,000 D. Disulfide
15   bonds form rapidly after the protein has been exported
from the cytoplasm. The expression of active protein on
the bacterial surface indicates that there is no
significant effect on the protein's ability to retain or
fold to its native form after membrane translocation.
20   This example also illustrates the versatility of the
method in that alkaline phosphatase is a relatively large
protein. In this example, tertiary and quaternary
structures are unaffected by the expression.

25   <u>Expression of Alkaline Phosphatase on *E. coli* Cell</u>
<u>Surface</u>

        Plasmid pTX101 was cut with *Eco*RI at the unique site
in the linker region between the OmpA and β-lactamase.
30   Subsequently the DNA was treated with the Klenow fragment
of DNA polymerase to create blunt ends. The *phoA* gene
coding for the sequence of alkaline phosphatase was
isolated from the plasmid pSWFII. A DNA fragment
containing the *phoA* gene was obtained by cutting pSWFII
35   with *Sba*I and then blunt-ended using the Kl now fragment.
The linearized pTX101 vector and th *phoA* gene fragment
were ligated overnight and the DNA was transformed into

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E. coli strain JM109. The new plasmid encoding the Lpp-OmpA-PhoA tripartite fusion was designated pTX1000. Exposure of the alkaline phosphatase on the surface of *E. coli* was tested by immunofluorescence microscopy using anti-alkaline phosphatase antibodies.

5

The degree of localization of alkaline phosphatase on the cell surface is expected to be enhanced in the strain JCB572 (Bardwell *et al.*, 1991) which is deficient in the gene for the *E. coli* periplasmic protein disulfide isomerase, DsbA, and in cultures incubated at sub-optimal growth temperatures, for example 24°C.

10

PROPHETIC EXAMPLE 6

15

The present example outlines the procedure contemplated as useful for expressing an antibody on the surface of *E. coli*. The antibody used for illustration is a catalysic antibody capable of catalysis in addition to binding its cognate antigen. Although this example is illustrated with antibody 37C4 against a particular hapten, other high affinity antibodies could be surface-expressed in a like manner. The example shows how the disclosed methods could be used to prepare a single-chain F_V, that is, a recombinant protein composed of a V_L chain linked to a V_H chain with a polypeptide linker. This particular ScF$_V$ is a catalytic antibody.

20

25

Expression of Single-Chain F_V Antibody on *E. coli* Cell Surface

30

Antibody 37C4 exhibits high binding against the hapten tris(4-methoxyphenyl)phosphonium (dissociation constant $>10^{-10} M^{-1}$. The antibody acts as a catalyst for the cleavage of various trityl ethers, increasing the reaction rate by about 200 fold compared to the uncatalyzed reaction in the absence of antibody. Total

35

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mRNA from the 37C4 hybridoma line is isolated and
purified by standard techniques (Ausubel et al. 1987).
The purified mRNA is used as a template for cDNA
synthesis using a polymerase chain amplification

5   technique (Sastry et al. 1989). The V_L and the V_H domains
of the 37C4 antibody are cloned using suitable primers
designed to introduce an in-frame EcoRI restriction site
at the N-terminus of the V_H and another one at the
carboxy terminus of the V_L for easy subcloning of the F_v

10 gene into the surface expression vector pTX101. Plasmid
pTX101 contains a unique EcoRI site located at the
downstream end of the DNA sequence for the OmpA domain
and immediately before the beginning of the ß-lactamase
gene. An Lpp-OmpA-scF$_v$ tripartite fusion is constructed

15 by digestion of pTX101 with EcoRI and ligation of the
scF$_v$ fragment. The resulting plasmid is transformed into
E. coli strain JM109. The presence of the single-chain
antibody on the cell surface allows the cells to bind to
a complex of tris(4-methoxyphenyl)phosphonium antigen

20 linked to the protein avidin. The antigen is linked to
avidin via its carboxy terminus by standard techniques
(Staros et al. 1986). Finally, avidin is detected by
immunofluorescence microscopy using anti-avidin
antibodies conjugated to fluorescein (obtained from

25 Vector Laboratories, Burlingame, CA. Cells expressing
the scF$_v$ fragment give a fluorescence signal whereas
control E. coli do not.

                        PROPHETIC EXAMPLE 7

30

      The present example outlines the procedure
contemplated as useful for the selection of antibodies
with high antigen binding affinity. The method is based
on a selection procedure for recombinant antibody

35 fragments on the surface of E. coli.

The method illustrated will overcome many of the
problems currently associated with display of antibody
molecules on phage surfaces. In particular, subcloning
will not be necessary for production, the number of
5   antibodies on the cell surface can be controlled and the
greater flexibility in the design of the expression
system will help ensure proper folding.

Selection of High Antigen Binding Affinity Antibodies
·10 Using a Cell Surface Display System

     BALB/C female mice (6-8 weeks old) are immunized
with the hapten tris(4-methoxyphenyl)phosphonium coupled
to Supercarrier (Pierce Chemical, Chicago, IL) dissolved
15 in Freund's complete adjuvant administered
intraperitoneally at a dose of 1 mg per animal. Follow
up injections are given intramuscularly once per week for
three weeks, rested 2 weeks and given a booster shot
before checking for antibody production. Incomplete
20 Freund's adjuvant is used for all injections subsequent
to the first injection.

     A library of single-chain F_v antibodies is
constructed using a polymerase chain reaction with total
25 spleen mRNA from the immunized mice (Clarckson et al.,
1991). The PCR primers are designed to introduce an in-
frame EcoRI restriction site at the N-terminus of the V_H
and another restriction site at the carboxy terminus of
the V_L for easy subcloning of the F_v gene into the surface
30 expression vector pTX101. Subsequently, the library DNA
is digested with EcoRI, a gene fragment of approximately
730 base pairs containing the entire scF_v gene is
identified. This DNA fragment is isolated and ligated to
EcoRI digested plasmid pTX101. The ligation mixture is
35 transformed into competent E. coli cells and
transformants are selected on LB plates containing the
antibiotic chloramphenicol. Plasmids in which the scF_v

-41-

is inserted in the correct orientation result in
expression of tripartite fusion proteins in th order
(from the amino terminus): Lpp-OmpA-scF$_v$. Colonies are
pooled from the plate and grown in rich media at 23°C to
5   allow efficient localization of the scF$_v$ to the cell
surface. Approximately 10^9 cells are diluted in buffer
to halt further growth and are loaded onto an affinity
column (approx 5 ml bed vol) containing the immobilized
hapten tris(4-methoxyphenyl)phosphonium. The column is
10   washed with Tris-HCl buffer, pH 7.0. Bound cells are
eluted by applying a linear gradient of the hapten.
Elution of the cells from the column is directly related
to the binding affinity of the exposed antibodies. This
results in enrichment of high-binding affinity antibodies
15   expressed on the surface of cells. The cells are
collected, grown and used to prepare antibodies.

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    The references listed below are incorporated herein
by reference to the extent that they supplement, explain,
provide a background for or teach methodology, techniques
and/or compositions employed herein.

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CLAIMS

1.   A tripartite chimeric gene for expression of a
desired polypeptide comprising:

5

        a targeting DNA sequence encoding a polypeptide
            capable of targeting a fusion polypeptide to a
            gram-negative cell outer membrane;

10   a DNA segment encoding a transmembrane amino acid
            sequence capable of transporting a heterologous or
            a homologous polypeptide through a gram-negative
            outer membrane; and

15   at least one DNA segment encoding a heterologous or
            a soluble homologous polypeptide

    wherein the chimeric gene is expressible in a gram
    negative bacterial cell when preceded by a functional
20 promoter,

                              or

        A recombinant vector capable of expressing a
25 heterologous or a soluble homologous polypeptide on a
    gram-negative bacterium outer membrane external surface,
    the vector comprising

        a targeting DNA sequence encoding a polypeptide
30   capable of targeting to the outer membrane of a
            gram negative bacterial cell;

        a transmembrane DNA sequence positioned adjacent to
            the targeting DNA sequence, said transmembrane
35   sequence encoding a polypeptide capable of
            transversing the cell outer membrane; and

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a DNA sequence encoding a heterologous or soluble
homologous polypeptide, said sequence b ing
positioned adjacent to said transmembrane
sequence.

5

2.   The recombinant vector of claim 1 wherein the
targeting DNA for an outer membrane protein precedes the
transmembrane sequence for said membrane protein.

10

3.   The chimeric gene or the recombinant vector of claim
1 wherein the targeting DNA sequence encodes an amino
acid sequence consisting of a signal sequence and at
15   least part of an outer membrane protein amino acid
sequence from a gram-negative bacterium.

4.   The chimeric gene or the recombinant vector of claim
20   1 further comprising a signal sequence having at least a
part of an amino acid sequence from an outer membrane
lipoprotein of *E. coli*.

25   5.   The chimeric gene or recombinant vector of claim 1
wherein the outer membrane protein amino acid sequence
comprises at least a part of *OsmB*, *TraT*, *NlpB*, *BlaZ*,
OmpA, LamB, OmpC, PhoE or *Pseudomonas* lipoprotein 1.

30
6.   The chimeric gene or the recombinant vector of claim
1 wherein the targeting DNA sequence encodes N-terminal
amino acid residues derived from nucleic acid of Figure 7
(SEQ ID NO:1) at base pair positions 1-87.

35

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7.   The chimeric gene or the recombinant vector of claim
1 wherein the encoded transmembrane protein sequence
comprises at least part of an outer membrane protein of
PhoE, LamB, OmpC, OmpF, OmpT, pilin or enterobactin
5   receptor or OmpA.

8.   The chimeric gene or the recombinant vector of claim
1 wherein the transmembrane sequence comprises an amino
10   acid sequence derived from nucleic acid sequence of
Figure 7 (SEQ ID NO:1) at base pairs 94-435.

9.   The chimeric gene or the recombinant vector of claim
15   1 wherein the gram negative cell is *Escherichia*,
Salmonella, Klebsiella, Erwinia, Shigella, Serratia or
Vibrio.

20   10.   The chimeric gene or the recombinant vector of claim
1 wherein the gram negative cell is a mutant *E. coli* or
Salmonella characterized as having an impaired ability to
form native disulfide bonds.

25
11.   The chimeric gene or the recombinant vector of claim
1 wherein the soluble homologous polypeptide is β-
lactamase or alkaline phosphatase.

30
12.   The chimeric gene or the recombinant vector of claim
1 wherein the heterologous polypeptide is a single chain
antibody or antibody fragment.

35
13.   An *Escherichia coli* or Salmonella transformant
prepared by transformation with the vector of claim 2.

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14. A method for obtaining a polypeptide anchored on the outer membrane surface of a gram-negative bacterial host cell comprising the steps:

5   preparing a recombinant vector according to claim 1;

           transforming a gram-negative bacterial cell with the
               recombinant vector;

10   culturing the transformed cells;

           screening for transformants; and

           culturing the transformant to obtain cells having a
15   polypeptide anchored on the outer membrane
               surface.

15. A method of eliciting an immune response in a
20   mammal, comprising the steps:

           a) selecting a polypeptide toward which an immune
               response is desired;

25   b) preparing a vector in accordance with claim 1
               wherein the heterologous or soluble homologous
               polypeptide is an antigenic polypeptide from an
               infectious agent; and

30   c) administering to a mammal a pharmaceutically
               acceptable preparation obtained from admixing
               cells transformed with the vector of step (b) with
               a vehicle suitable for administration in a mammal.

35

16. A heterologous or soluble homologous polypeptide
expressed by a gram-negative cell transformed with a

recombinant vector comprising the chimeric gene of claim 1.

5   17.   A kit useful for transforming gram-negative bacterial host cells comprising:

        a transporter being compartmentalized to receive one
        or more container means in close confinement
10   therein; and

        a first container means comprising at least one
        recombinant vector in accordance with claim 1, the
        vector being suitably aliquoted into the
15   container.

18.   The kit of claim 17 wherein the vector is in accordance with SEQ ID NO:1.

20

19.   A recombinant vector in accordance with SEQ ID NO: 1.

25

20.   A method for removing contaminants from fluids comprising admixing a gram-negative bacterium transformed with the recombinant vector of claim 1 with a contaminant-containing fluid wherein the encoded
30 heterologous or soluble homologous polypeptide located on the bacterial surface binds selectively with the contaminant.

35 21.   The method of claim 20 wherein the encoded polypeptide is metallothionein or a single chain antibody or antibody fragment.

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22. The method of claim 20 further comprising cross-
linking the transformed bacterial cells before admixing
with the contaminant.

5

23. An immobilized enzyme system comprising an enzyme
expressed on the outer membrane surface of a gram-
negative bacterial host cell transformed with the
recombinant vector of claim 1 wherein the encoded
10   heterologous or soluble homologous polypeptide comprises
a biocatalytically active polypeptide.

24. The immobilized enzyme system of claim 23 wherein
15   the encoded polypeptide comprises a catalytic antibody.

25. A method of selecting high affinity recombinant
antibodies or antibody fragments, comprising the steps:
20
     a) constructing a library of genes encoding at least
        one antibody or antibody fragment;

     b) preparing vectors in accordance with claim 1
25   wherein the heterologous or soluble homologous
        polypeptide comprises one or more genes of said
        library;

     c) transforming a gram-negative bacterial cell using
30   the recombinant vectors;

     d) culturing said transformed cells; and

     e) binding the expressed antibodies or antibody
35   fragments to a suitable antigen wherein cells
        expressing high affinity antibodies or antibody
   fragments are selected.
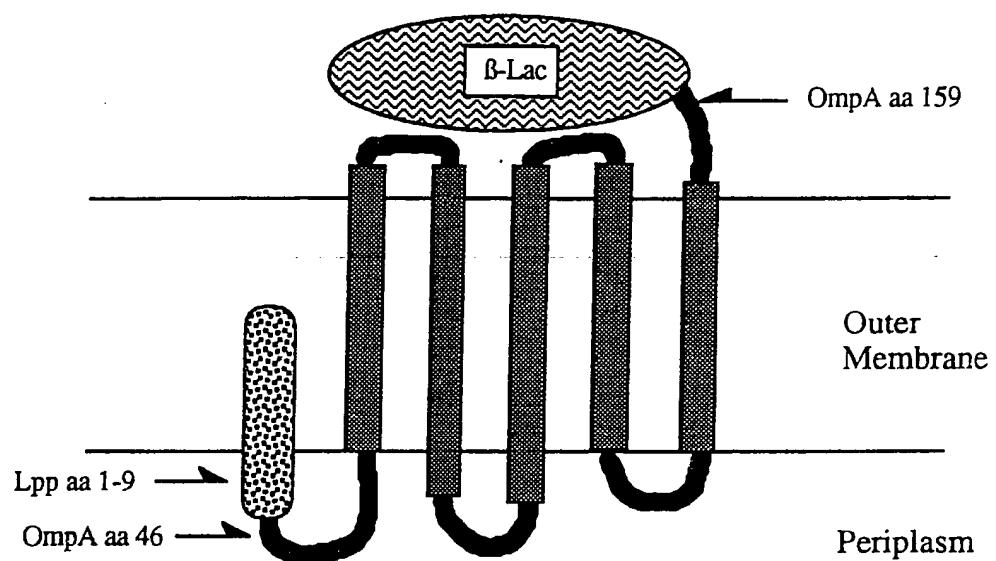
FIG. 1
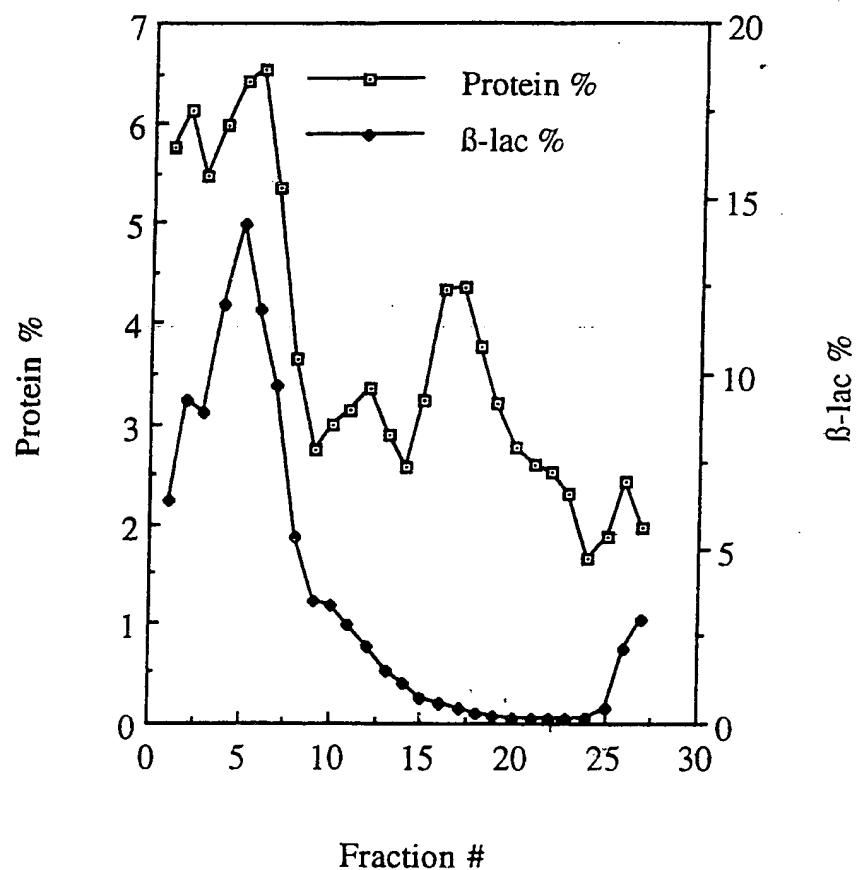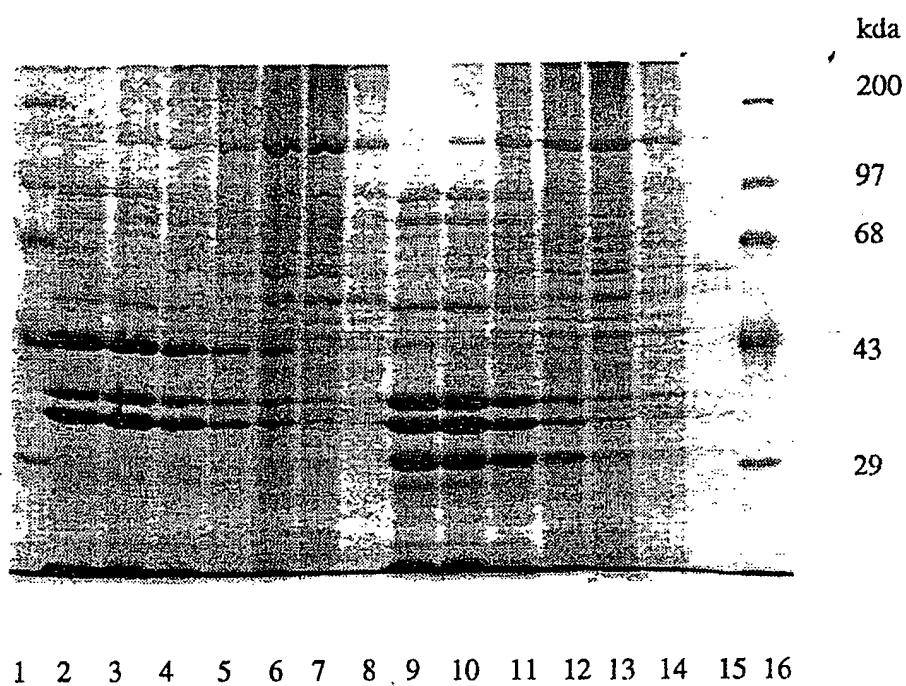
FIG. 2

FIG. 3A

kda
106
80

49

32

1   2   3   4   5   6   7   8
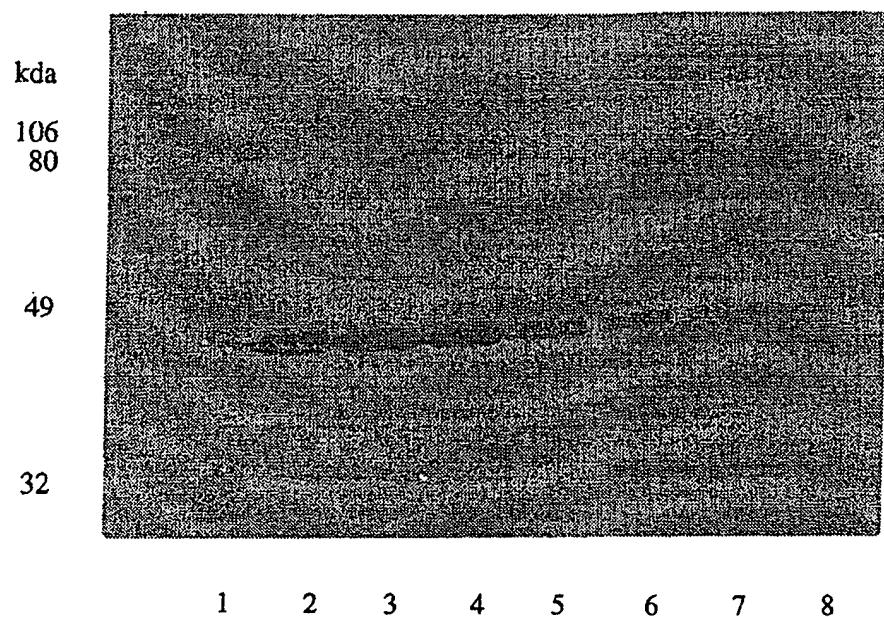
FIG. 3B
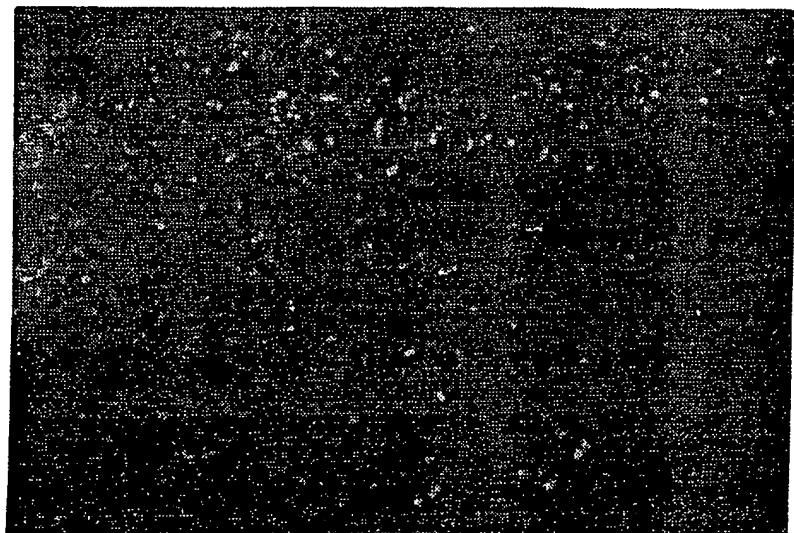
FIG. 4A
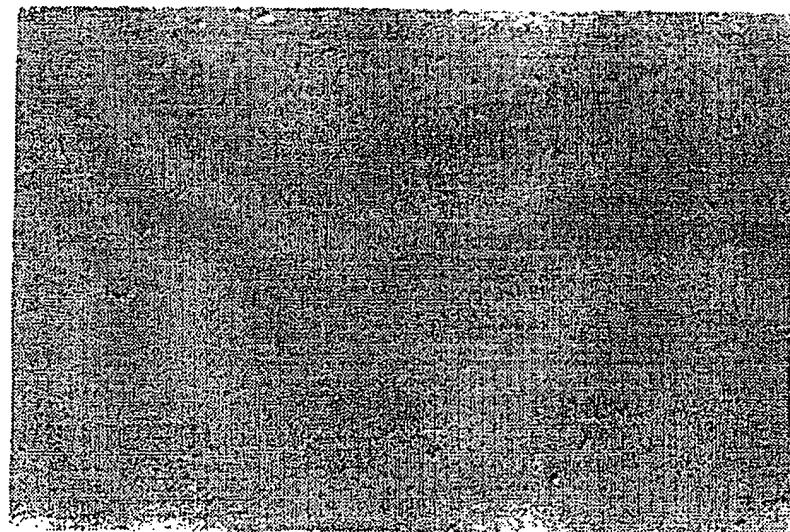
FIG. 4B
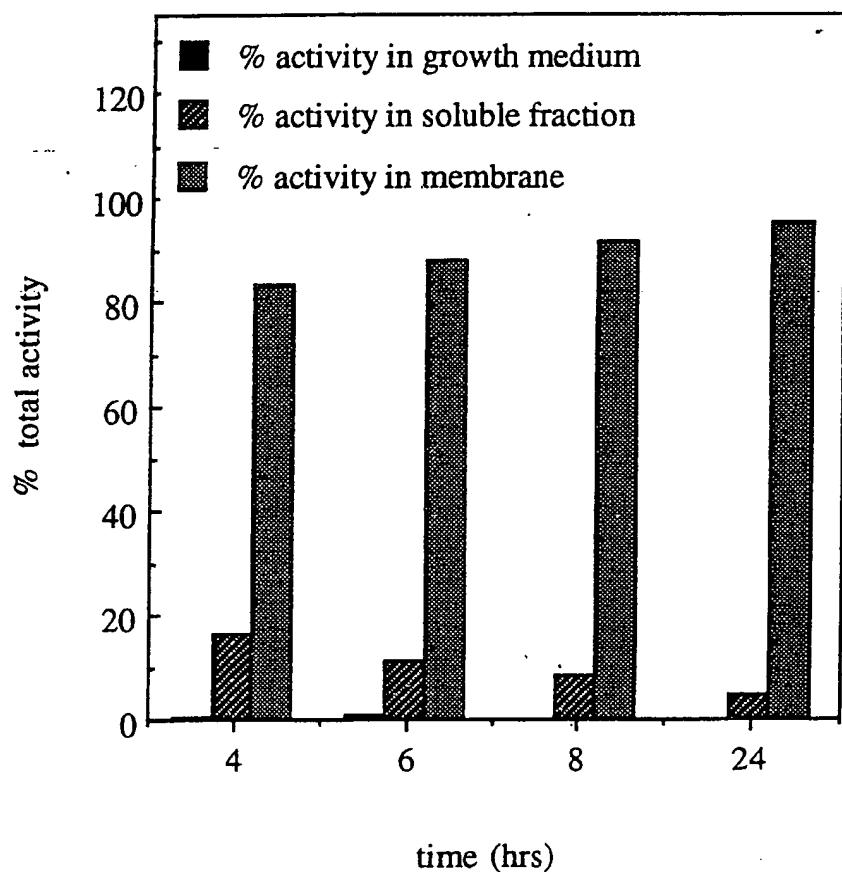
FIG. 5
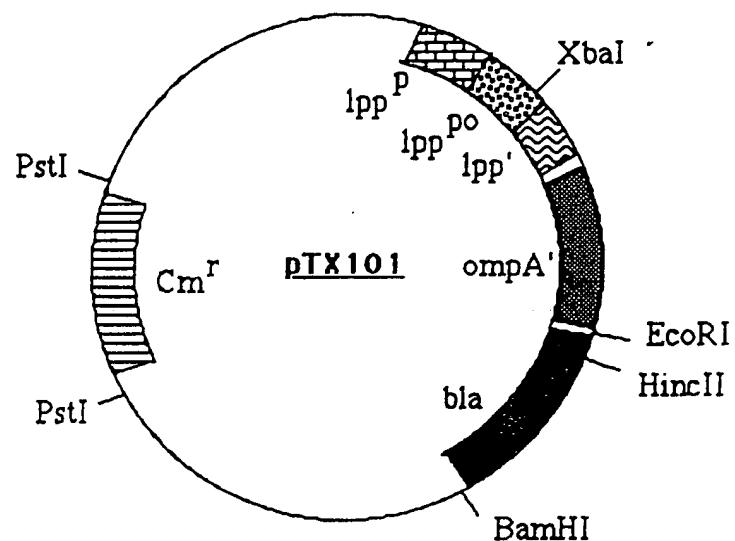
FIG. 6

FIG. 7A

```
ATGAAAGCTA CTAAACTGGT ACTGGGCGCG GTAATCCTGG GTTCTACTCT GCTGGCAGGT   60
TGCTCCAGCA ACGCTAAAAT CGATCAGGGA ATTAACCCGT ATGTTGGCTT TGAAATGGGT  120
TACGACTGGT TAGGTCGTAT GCCGTACAAA GGCAGCGTTG AAAACGGTGC ATACAAAGCT  180
CAGGGCGTTC AACTGACCGC TAAACTGGGT TACCCAATCA CTGACGACCT GGACATCTAC  240
ACTCGTCTGG GTGGCATGGT ATGGGCGTGCA GACACTAAAT CCAACGTTTA TGGTAAAAAC  300
CACGACACCG GCGTTTCTCC GGTCTTCGCT GGCGGTGTTG AGTACGCGAT CACTCCTGAA  360
ATCGCTACCC GTCTGGAATA CCAGTGGACC AACAACATCG GTGACGCACA CACCATCGGC  420
ACTCGTCCGG ACAACGGAAT TCCGGGTCAC CCAGAAAACGC TGGTGAAAGT AAAAGATGCT  480
GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC  540
CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA  600
TGTGGCGCGG TATTATCCCG TGTTGACGCC GGGCAAGAGC AACTCGGTCG CCGCATACAC  660
TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC  720
ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAAACAC TGCCGGCCAAC  780
TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG  840
GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC  900
GAGCGTGACA CCACGATGCC TGCAGCAATG TGCAGCAACGT TGCGCAAACT ATTAACTGGC  960
GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT 1020
```

FIG. 7B

GCAGGACCAC TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTCGTGA TAAATCTGGA 1080

GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC 1140

CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG 1200

ATCGCTGAGA TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA 1260

TATATACTTT AGA   1273

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FIG.8A

```
ATG AAA GCT ACT AAA CTG GTA CTG GGC GCG GTA ATC CTG GGT TCT ACT CTG  51
Met Lys Ala Thr Lys Leu Val Leu Gly Ala Val Ile Leu Gly Ser Thr Leu
1               5                   10                  15

CTG GCA GGT TGC TCC AGC AAC GCT AAA ATC GAT CAG GGA ATT AAC CCG TAT  102
Leu Ala Gly Cys Ser Ser Asn Ala Lys Ile Asp Gln Gly Ile Asn Pro Tyr
            20                  25                  30

GTT GGC TTT GAA ATG GGT TAC GAC TGG TTA GGT CGT ATG CCG TAC AAA GGC  153
Val Gly Phe Glu Met Gly Tyr Asp Trp Leu Gly Arg Met Pro Tyr Lys Gly
        35                  40                  45              50

AGC GTT GAA AAC GGT GCA TAC AAA GCT CAA CTG ACC GCT AAA  204
Ser Val Glu Asn Gly Ala Tyr Lys Ala Gln Leu Thr Ala Lys
    55                  60                  65

CTG GGT TAC CCA ATC ACT GAC GAC CTG GAC ATC TAC ACT CGT CTG GGT GGC  255
Leu Gly Tyr Pro Ile Thr Asp Asp Leu Asp Ile Tyr Thr Arg Leu Gly Gly
        70                  75                  80              85

ATG GTA TGG CGT GCA GAC ACT AAA TCC AAC GTT TAT GGT AAA AAC CAC GAC  306
Met Val Trp Arg Ala Asp Thr Lys Ser Asn Val Tyr Gly Lys Asn His Asp
        90                  95                  100
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SUBSTITUTE SHEET

FIG. 8B

```
ACC GGC GTT TCT CCG GTC TTC GCT GGC GGT GTT GAG TAC GCG ATC ACT CCT 357
Thr Gly Val Ser Pro Val Phe Ala Gly Gly Val Glu Tyr Ala Ile Thr Pro
             105                 110                 115

GAA ATC GCT ACC CGT CTG GAA TAC CAG TGG ACC AAC AAC ATC GGT GAC GCA 408
Glu Ile Ala Thr Arg Leu Glu Tyr Gln Trp Thr Asn Asn Ile Gly Asp Ala
         120                 125                 130             135

CAC ACC ATC GGC ACT CGT CCG GAC AAC GGA ATT CCG GGT CAC CCA GAA ACG 459
His Thr Ile Gly Thr Arg Pro Asp Asn Gly Ile Pro Gly His Pro Glu Thr
             140                 145                 150

CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC 510
Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr
         155                 160                 165             170

ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA 561
Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu
             175                 180                 185

GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA 612
Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val
             190                 195                 200
```

FIG. 8C

```
TTA TCC CGT GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT 663
Leu Ser Arg Val Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr
205                     210                  215                  220

TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG 714
Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
            225                  230                  235

GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT 765
Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser Asp
        240                  245                  250                  255

AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA 816
Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu
            260                  265                  270

ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG 867
Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu Asp Arg Trp
            275                  280                  285

GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG 918
Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg Asp Thr Thr Met
        290                  295                  300                  305
```

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FIG. 8D

CCT GCA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT 969
Pro Ala Ala Met Ala Thr Thr Ala Thr Leu Arg Lys Leu Leu Thr Gly Glu Leu Leu
                                              315   320

ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT 1020
Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu Ala Asp Lys Val
325   330   335   340

GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT CGT GAT 1071
Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile Arg Asp
              345   350   355

AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG 1122
Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly
360   365   370

CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG 1173
Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln
375   380   385   390

GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG 1224
Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu
              395   400   405

ATT AAG CAT TGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGA   1273
Ile Lys His Trp
410   412

FIG. 9
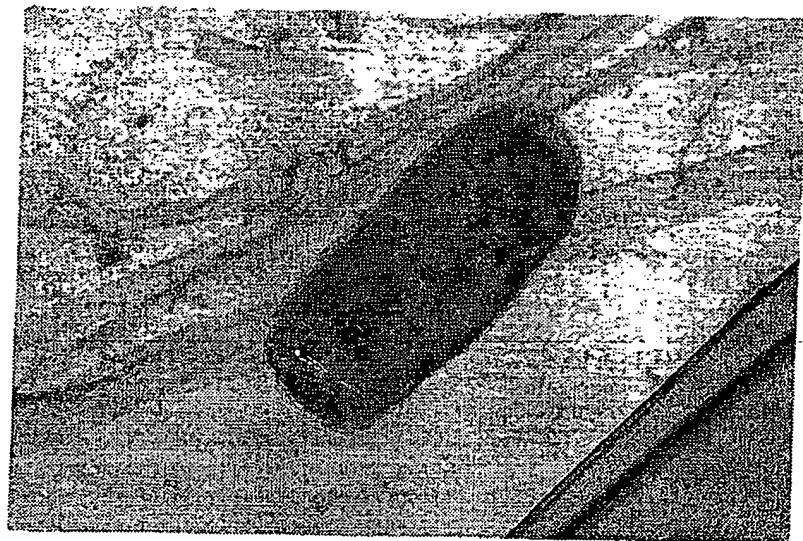
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/21, 15/00; 15/12; A61K 37/02, 39/395

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 530/324, 350, 387.1, 399; 435/7.2, 7.32, 7.35, 7.37, 69.7, 69.8, 172.1, 252.1, 252.3, 252.33, 320.1; 514/2, 12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: Escherichia, coli, surface, outer membrane, gram negative, anchor, surface display

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Volume 55, Number 4, issued April 1989, G. Thiry et al., "Cloning of DNA Sequences Encoding Foreign Peptides and Their Expression in the K88 Pili", pages 984-993, see entire document.	1,7,9,13-14, 17 2-3;10;15-16
X Y	JOURNAL OF BACTERIOLOGY, Volume 171, Number 11, issued November 1989, C.K. Murphy et al., "Export of FepA::PhoA Fusion Proteins to the Outer Membrane of Escherichia coli K-12", pages 5894-5900, see entire document.	1-3, 7, 9,11,13, 14,17 15-16
X Y	MOLECULAR MICROBIOLOGY, Volume 4, Number 7, issued 1990, M.G. Komacker et al., "The normally periplasmic enzyme β-lactamase is specifically and efficiently translocated through the Escherichia coli outer membrane when it is fused to the cell-surface enzyme pullulanase", pages 1101-1109, see entire document.	1,9,11,13-14,17 2-3,7,15-16

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 1992	

Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MARIANNE PORTA ALLEN
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	THE EMBO JOURNAL, Volume 5, Number 11, issued 1986, A. Charbit et al., "Probing the topology of a bacterial membrane protein by genetic insertion of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface", pages 3029-3037, see entire document.	1,5,7,9,13-14,17 2-3,15-16,23-25
X Y	GENE, Volume 59, issued 1987, M. Agterberg et al., "Use of outer membrane protein PhoE as a carrier for the transport of a foreign antigenic determinant to the cell surface of Escherichia coli K-12", pages 145-150, see entire document.	1,5,7-9,13-14 2-3,15-16,23-25
X Y	RES. MICROBIOL., Volume 141, issued 1990, S. Pistor et al., "OmpA-Haemagglutinin Fusion Proteins for Oral Immunization with Live Attenuated Salmonella", pages 879-881, see entire document.	1,5,8-9,13-14,17,23-25 2-3,10,15-16
X Y	THE EMBO JOURNAL, Volume 9, Number 6, issued 1990, T. Klauser et al., "Extracellular transport of cholera toxin B subunit using Neisseria IgA protease β-domain: conformation-dependent uter membrane translocation", pages 1991-1999, see entire document.	1,9,12,14,17 15-16
X Y	GENE, volume 83, issued 1989, F.A. Jacobs et al., "Human metallothionein-II is synthesized as a stable membrane-localized fusion protein in Escherichia coli", pages 95-103, see entire document.	1-4,9,13-14,17 6,15-16,20-22
X,P Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 87, Number 7, issued 01 April 1992, J.A. Francisco et al., "Transport and anchoring of β-lactamase to the external surface of Escherichia coli", pages 2713-2717, see entire document.	1-9,11,13-14,16-19 10,12,15,20-25
Y,P	ABSTR. PAP. AM. CHEM. SOC., Volume 203, Part 1, Abstract BIOT 116, issued 1992, J.A. Francisco et al., "Developments and applications of expression systems for the anchoring of proteins on the surface of Gram-negative bacteria", see entire abstract.	1-25

A. CLASSIFICATION OF SUBJECT MATTER:
 US CL :

536/27; 530/324, 350, 387.1, 399; 435/7.2, 7.32, 7.35, 7.37, 69.7, 69.8, 172.1, 252.1, 252.3, 252.33, 320.1; 514/2, 12